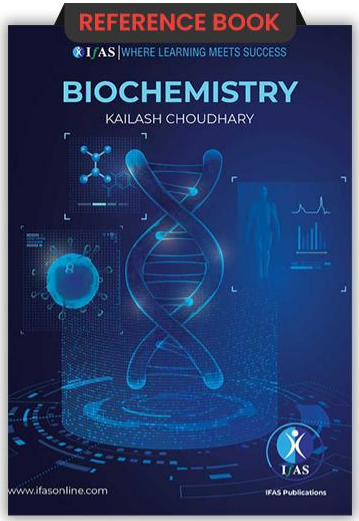
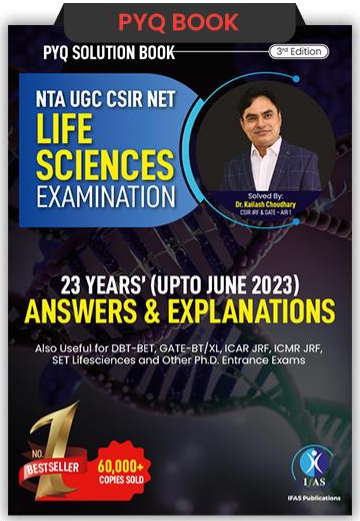
"There's can be no rainbow without a little rain"

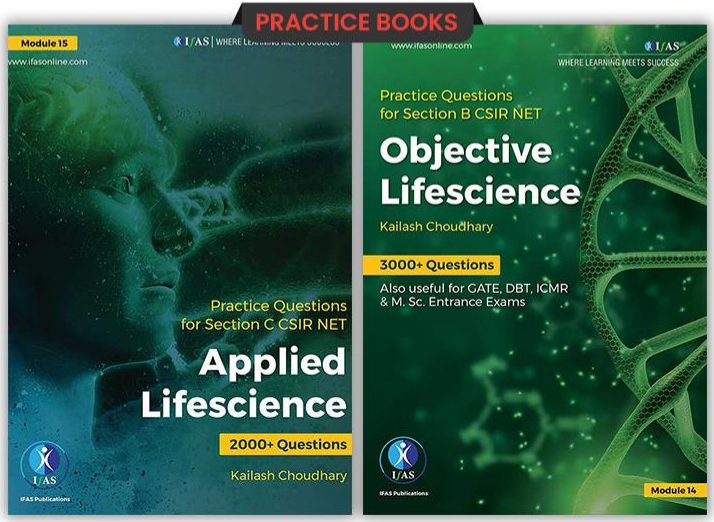
CSIR NET – Life Science

Unit 1: Biochemistry

12 Enzyme Kinetics & Inhibition







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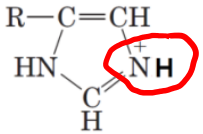
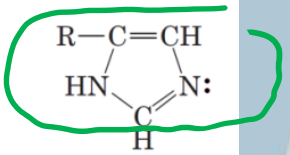
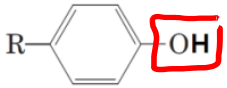
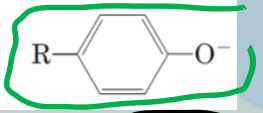
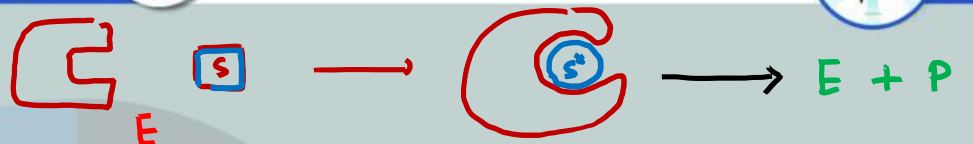
Points to be covered in this Lecture

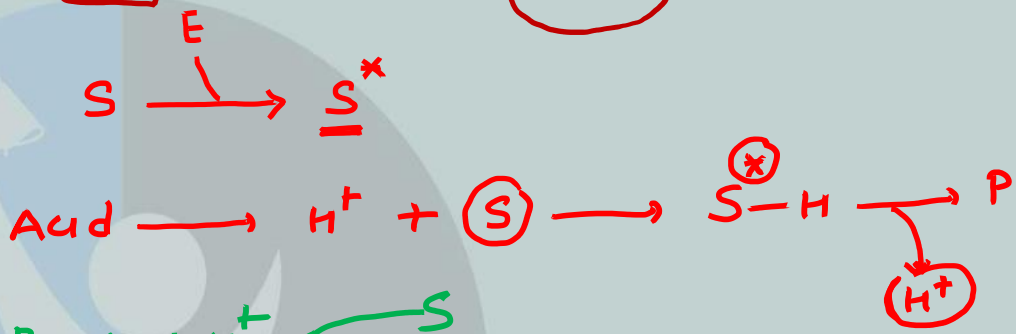
- Catalytic Strategies
- Michalis Menten Kinetics
- Km, Kcat and Vmax
- LB Plot, HW Plot, EF Plot
- Irreversible Inhibitors
- Reversible inhibitors
- Dixon plot

Catalytic Strategies:

I. General Acid-Base Catalysis

Amino acid residues	General acid form (proton donor)	General Base form (proton acceptor)
Glu, Asp	R—COOH	R—COO⁻
Lys, Arg	R—⁺NH₃	R—NH₂
Cys	R—SH	R—S⁻
His		
Ser	R—OH	R—O⁻
Tyr		

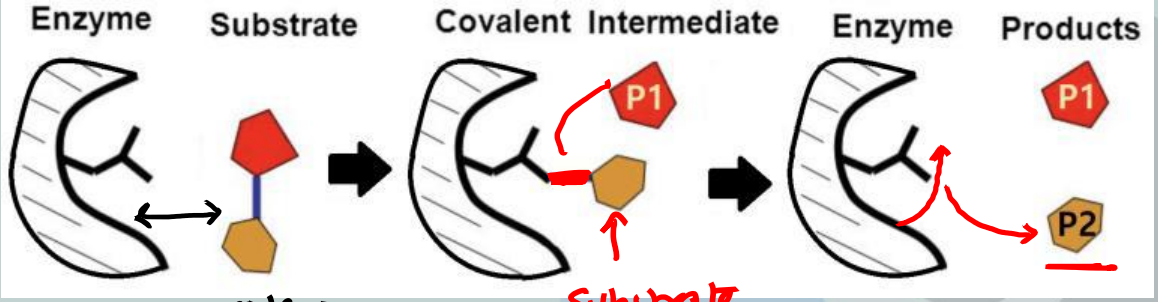




eg Lysozyme
Trypsin
Chymotrypsin

Specific Acid-Base Catalysis: Water → H^+ donor or acceptor

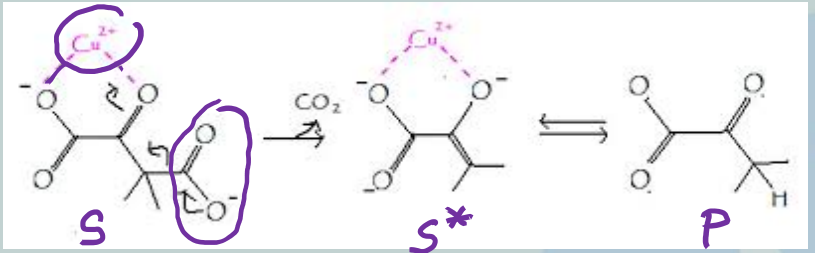
2. Covalent Catalysis



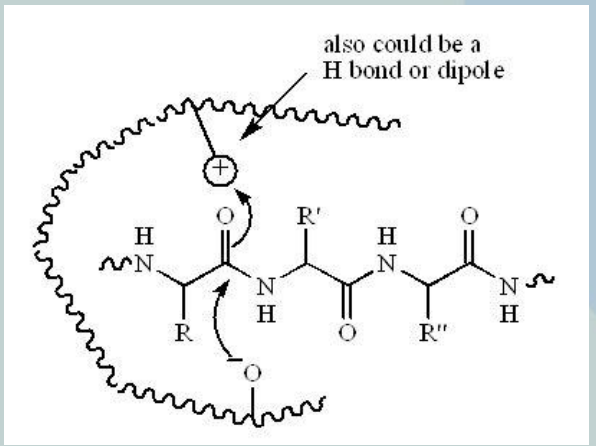
Ser - Asp - His

- Trypsin
- Chymotrypsin
- Hydrolytic Enzyme
- Recombinase

3. Metal Ion Catalysis : Mg^{2+}, Ca^{2+}, Fe^{2+} ..... neutralize -ve charge of substrate so that transition state can be achieved.



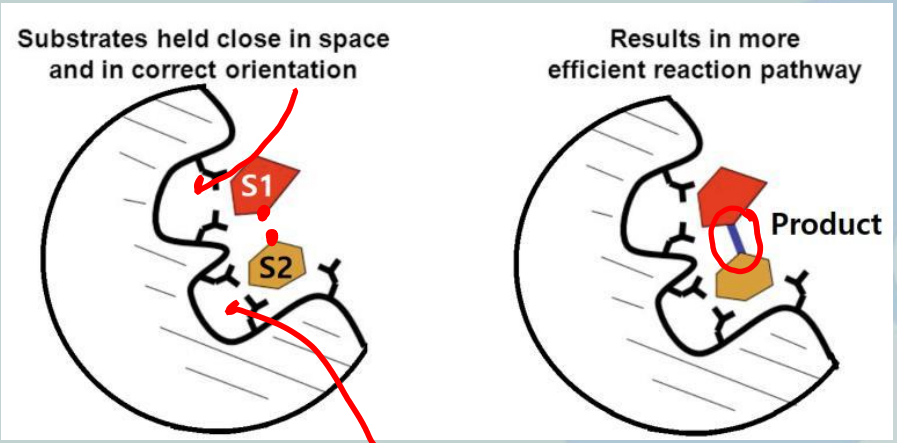
4. Electrostatic Catalysis

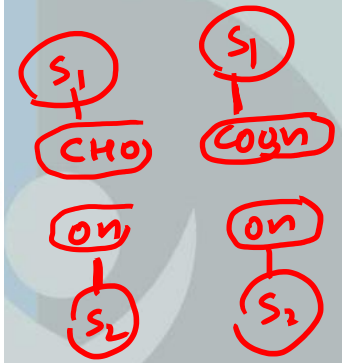


- D= ↓
- Electrostatic interaction = ↑
- H- bonding = ↑

betⁿ Enzyme & substrate

5. Catalysis through Proximity and Orientation Effects



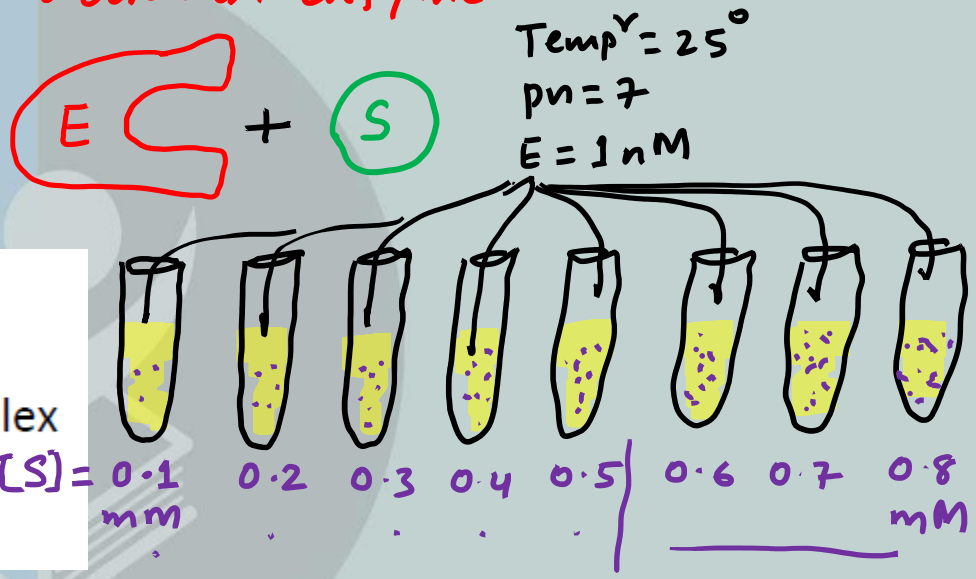


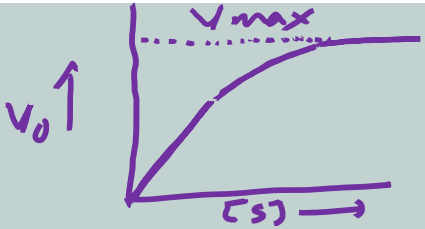
ENZYME KINETICS : Michelis-Menten Kinectic

• classical enzyme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Where, S is the substrate
E is the enzyme
ES is the enzyme-substrate complex
P is the product
k_1, k_{-1}, and k_2 are rate constants





$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$

$$V_0 = \frac{V_{max}\,[S]}{K_m + [S]}$$

Where, Vo = initial reaction velocity

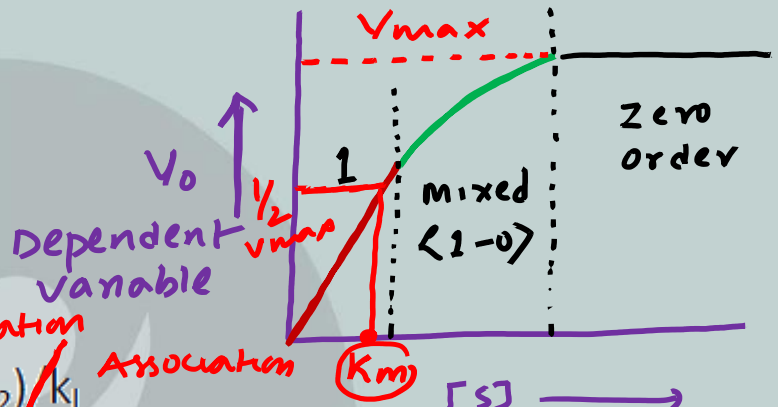
Vmax = maximal velocity

Km = Michaelis constant = $(k_{-1} + k_2)/k_1$

[S]= substrate concentration

Dissociation

Association



$V_{max} \propto E_{Total}$ $\quad V_{max} = K_2 E_T$

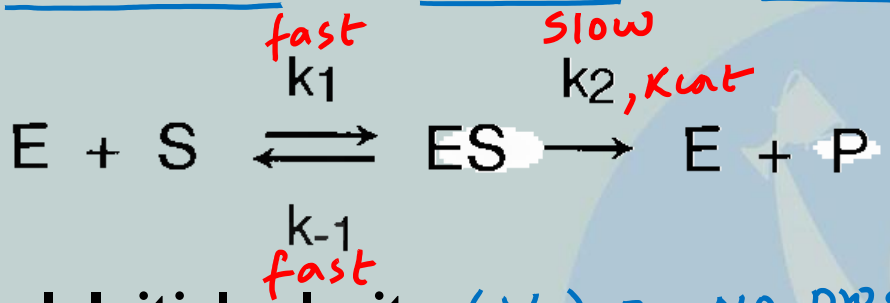
$V_0 \propto [S]$

Km is also some constant substrate conc^n. $\quad V_0 = \frac{1}{2} V_{max}$

$$V_0 = V_{max}\frac{[S]}{K_m + [S]}$$

$$\frac{V_0}{V_{max}} = \frac{[S]}{K_m + [S]}$$

Assumptions of Michaelis and Menten Equation



$$\frac{V_0}{V_{max}} = \frac{[S]}{K_m + [S]}$$

1. **Initial velocity** (V_0) = No product, all rxn will proceed forward
2. **[S]>>[E]** [S] = 0.1 mM to 1 mM >> [E] = 1 nM
3. **ES dissociation is insignificant** : [ES] conc^{r} remains constant
4. **Steady-state assumption:** Formation of ES = Dissociation of ES
5. **Rate limiting step is K2** or (slowest) Kcat Turnover number

$$K_1 = K_{-1} + K_2$$

$K_{-1} >>> K_2$

$$V_0 = V_{max} \frac{(S)}{K_m + (S)}$$

What is Km and Ks?

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} E + P$$

- Dissociation constant for ES complex

$$K_m = \frac{\text{Dissociation rate constant}}{\text{Association rate constant}} = \frac{K_{-1} + K_2}{K_1}$$

$$K_s = \frac{K_{-1}}{K_1}$$

- Substrate concentration where Vo=0.5 Vmax

$$\frac{V_o}{V_{max}} = \frac{(S)}{K_m + (S)}$$

Suppose $V_o = \frac{1}{2} V_{max}$

$$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

$$K_m + 1[S] = 2[S]$$

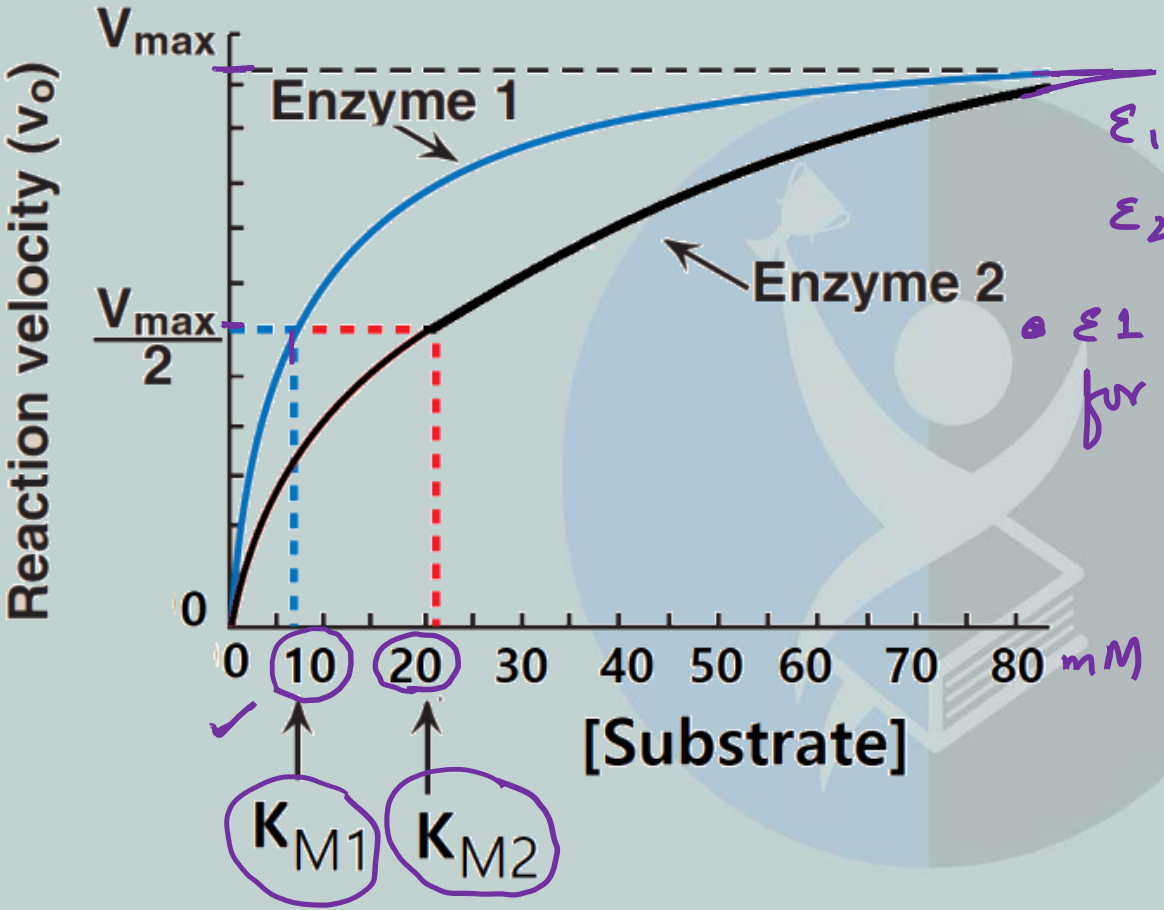
$$K_m = 2[S] - 1[S]$$

$$K_m = [S]$$

$[S] = K_m$	$V_o = \frac{1}{2} V_{max}$
$[S] < K_m$	$V_o < \frac{1}{2} V_{max}$
$[S] > K_m$	$V_o > \frac{1}{2} V_{max}$

- High Km means low affinity of enzyme for its substrate
- **K_M does not vary with the concentration of enzyme or substrate**

$[S] = \uparrow$ $V_o = \uparrow$ $[E] = \uparrow$ $V_{max} = \uparrow$



$E_1 = K_{m_2} = 10\ mM$

$E_2 = K_{m_2} = 20\ mM$

- E_1 has higher affinity for its substrate

Apply your Mind

$V_0 = V_{max} \frac{(S)}{K_m + (S)}$

$V_0 = 100 \frac{\mu mol}{min} \times \frac{10\ mM}{10\ mM + 10\ mM}$

$V_0 = 100 \times \frac{10}{20} = 50 \frac{\mu mol}{min}$

The enzyme alkaline phosphatase was tested for its catalytic activity using the substrate para-nitrophenylphosphate. The Km obtained was 10 mM and Vmax was 100 umol/min. Which one of the following options represents the initial velocity of the reaction at a substrate concentration of 10 mM?

(1) 50 µmol/min
(2) 100 µmol/min
(3) 500 µmol/min
(4) 20 µmol/min

$[S] = K_m \quad V_0 = 0.5\ V_{max}$

$[S] > K_m \quad V_0 > 0.5\ V_{max}$

$[S] < K_m \quad V_0 < 0.5\ V_{max}$

$10mM = 10mM$

$V_0 = 0.5 \times 100 \frac{\mu mol}{min}$

$= 50$

Apply your Mind

$$V_0 = \frac{V_{max}(S)}{1Km + (S)}$$

What is the fold difference between Vo at [S] = ½ Km and Vo at [S] = 2Km where Vo is the initial velocity of an enzyme catalyzed reaction, [S] is substrate concentration and Km is the Michaelis constant?

(1) 2 Fold
(2) 4 Fold
(3) 6 Fold
(4) 8 Fold

Rx 1

$V_0 = ?$

$[S] = 0.5\ Km$

$$V_0 = V_{max}\frac{0.5Km}{1Km + 0.5Km}$$

$$= V_m\frac{0.5Km}{1.5Km}$$

$$= V_m \times 1/3$$

$$V_0 = 0.33\ V_{max}$$

Rx 2

$V_0 = ?$

$[S] = 2\ Km$

$$V_0 = V_{max}\frac{2Km}{1Km + 2Km}$$

$$V_0 = V_{max}\frac{2Km}{3Km}$$

$$V_0 = \frac{2}{3} V_{max}$$

$$V_0 = 0.66\ V_{max}$$

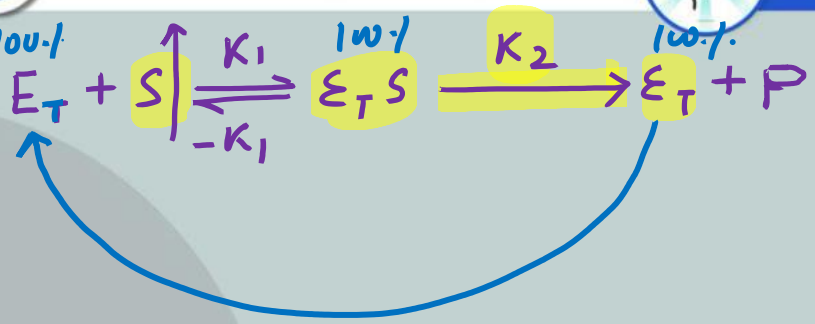
What is Kcat or turnover number?

$V_{max} = K_2 E_T$

$V_{max} = K_{cat} \varepsilon_T$

$K_{cat} = V_{max}/E_T$

- V_{max}: collective speed
- K_{cat}: Speed of single enzyme
- ε_T: Total Enzyme



V_{max} = M/sec

ε_T = M

$V_o = \frac{V_{max}(S)}{K_m + (S)}$

K_{cat} is more : enzyme is fast

K_{cat} is less : enzym is slow

K_{cat} is constant, donot vary with substrate conc^n or enzyme conc^n.

Fast and slow enzymes

Per second

Kcat or K_2 or

i) Enzyme	ii) Turnover number
Catalase ✓	4.0×10^7
Carbonic anhydrase ✓	1.0×10^6
Acetylcholine esterase ✓	2.5×10^4
Lactate dehydrogenase	1.0×10^3
Urease ✓	1.0×10^4
Chymotrypsin ✓	100.0
Rubisco slow	→ 3.0
Lysozyme slow	→ 0.5

*For Ribozymes and restriction endonuclease, it is expressed per minute

Very slow

Turnover (Kcat) = $\frac{V_{max}}{E_T}$

$= \frac{M/sec}{M}$

Kcat = 1/sec

Kcat = sec^{-1}

First order rate constant

Specificity constant or catalytic efficiency

Kcat/Km is rate constant that refers to properties and reactions of free enzyme and free substrate

Enzyme	Substrate	K_M (M)	k_{cat} (s^{-1})
Acetylcholinesterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}
Carbonic anhydrase	CO_2	1.2×10^{-2}	1.0×10^{6}
Superoxide dismutase	Superoxide ion ($O_2^{-\cdot}$)	3.6×10^{-4}	1.0×10^{6}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}

Kcat / Km

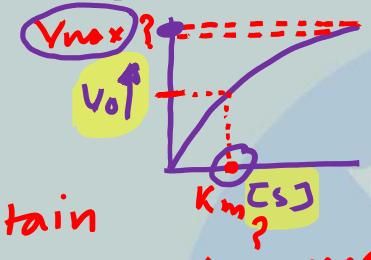
$1.4 \times 10^4 / 9.5 \times 10^{-5} \cong \ldots \times 10^8$

$10^6 / 1.2 \times 10^{-2} \cong \ldots \times 10^8$

Kcat/Km of 2 X 10^8 for enzyme indicates that the value is close to diffusion controlled rate of encounter

Line-weaver-Burk equation and Plot

$V_0 = \frac{V_{max}(S)}{K_m + (S)}$



Rectangular Hyberbolic curve

Difficult to obtain Vmax & Km precisely by MM plot

convert it into straight line $Y = mx + c$

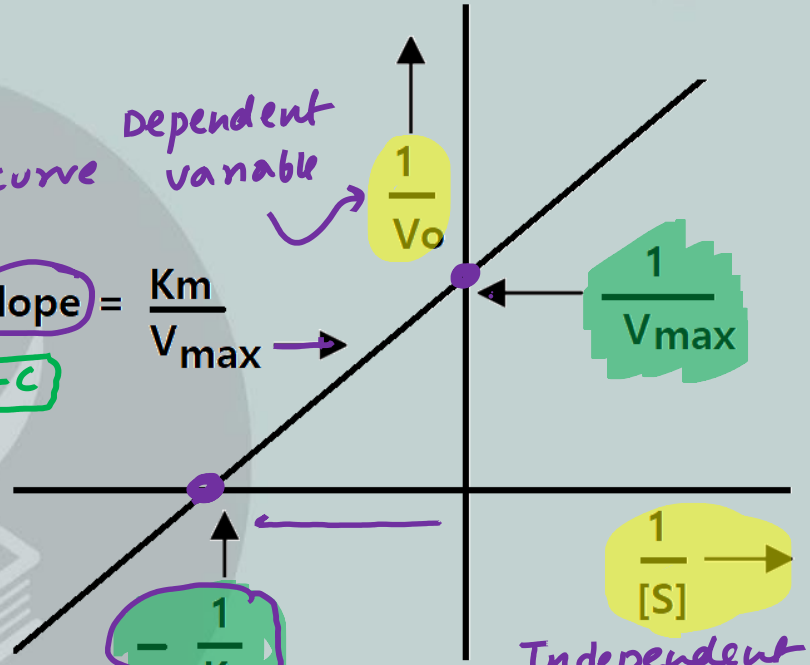
Double Reciprocal of MM eqn to obtain straight line

$\frac{1}{V_0} = \frac{K_m + [S]}{V_{max}[S]}$

$\frac{1}{V_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{[S]}{V_{max}} \cdot \frac{1}{(S)}$

$\frac{1}{V_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{(S)} + \frac{1}{V_{max}}$

$Y = m x + c$



The Hanes-Woolf plot

LB Burk equation multiplied by [S]

$$\frac{[S]}{v_0} = \frac{1}{Vmax} \cdot [S] + \frac{K_M}{Vmax}$$

$$Y = m \cdot x + c$$

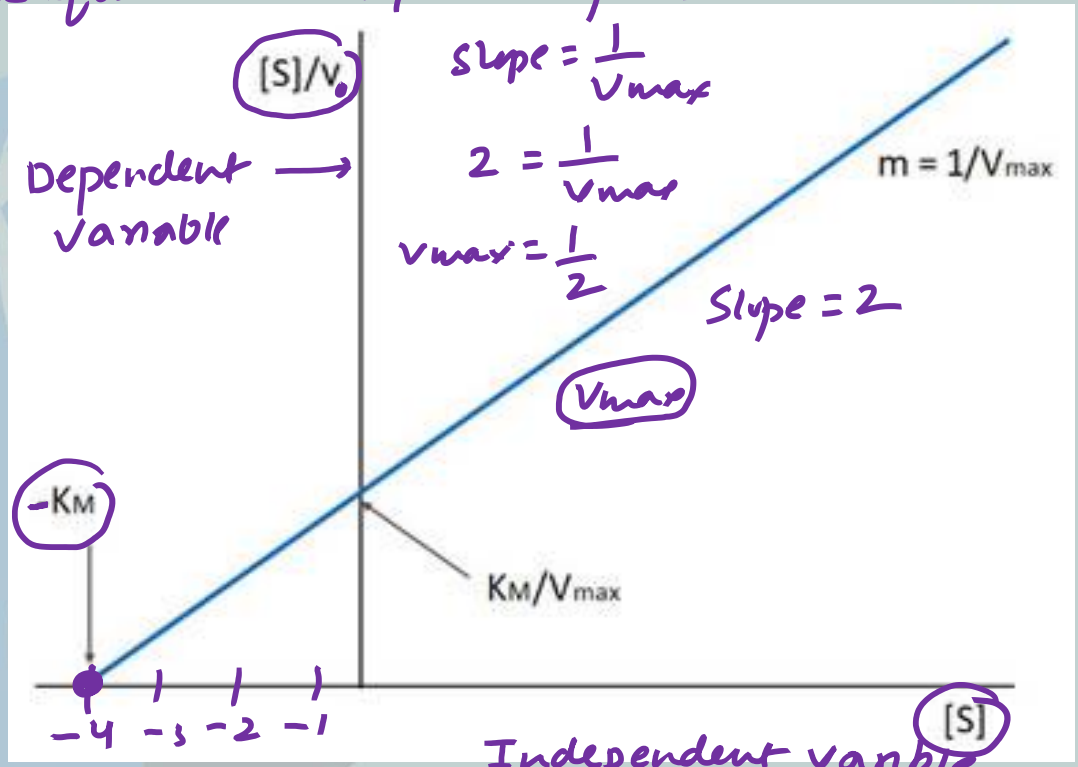
Slope = $\frac{1}{Vmax}$

Reciprocal of slope = Vmax

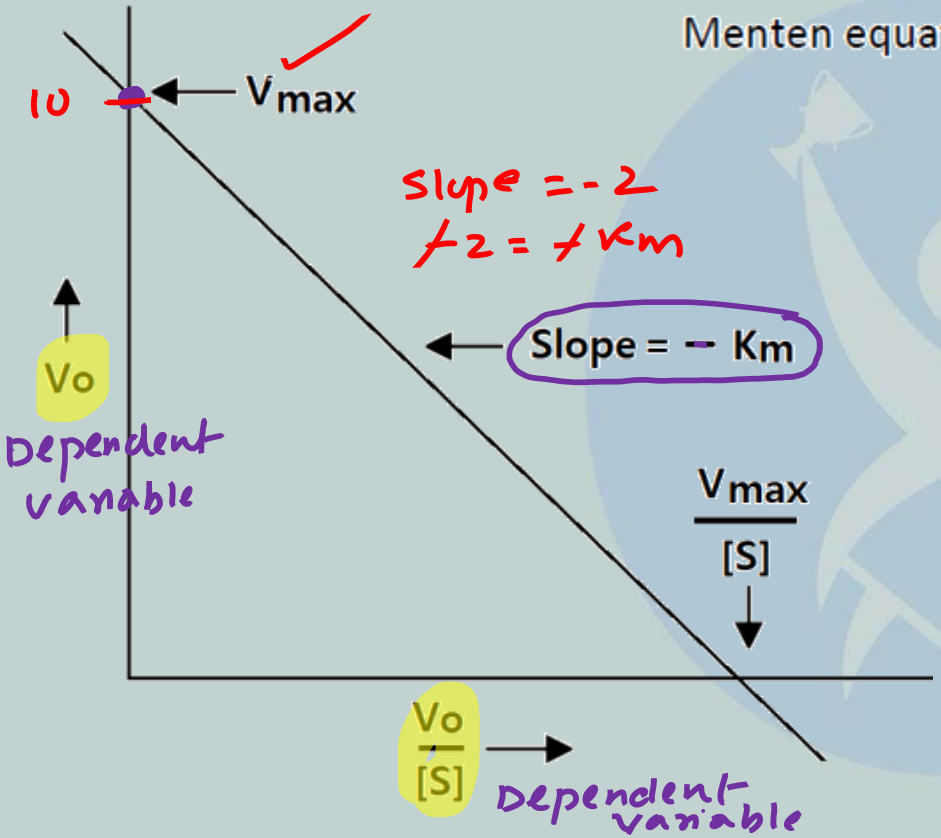
x-axis intercept = Km

$\neq Km = \neq 4$

$Km = 4$



Eadie-Hofstee Plot (Imp)



The Eadie-Hofstee's equation is derived from Michaelis-Menten equation as shown below,

$$V_0 = \frac{V_{max}\,[S]}{K_M + [S]}$$

$V_0 (K_m + [S]) = V_{max} [S]$

$V_0 K_m + V_0 [S] = V_{max} [S]$

Divide it by $[S]$

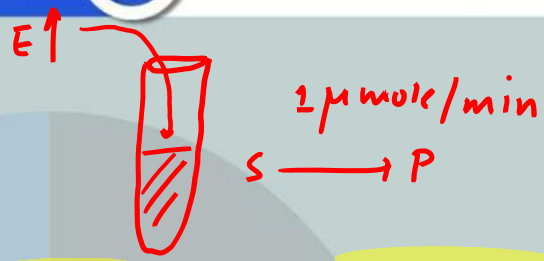
$$\frac{V_0 K_m}{[S]} + \frac{V_0 [S]}{[S]} = \frac{V_{max} [S]}{[S]}$$

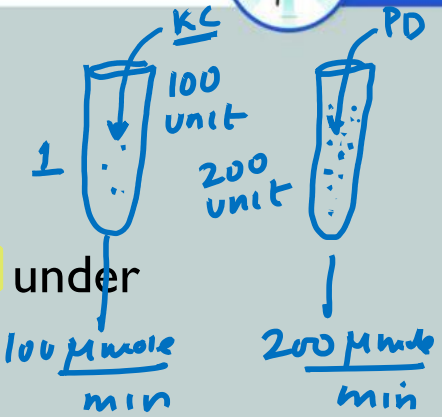
$$\frac{V_0}{(S)} \cdot K_m + V_0 = V_{max}$$

$$V_0 = -K_m \cdot \frac{V_0}{(S)} + V_{max}$$

$y = -m \cdot x + c$

Expression of enzyme activity





1 UNIT:

one µmole of substrate transformed or *product* formed *per Minute* under defined conditions (*µmole/min*)

$1 \text{ unit} = 1 \frac{\mu\text{mole}}{\text{min}}$

1 Ketal:

$1 \text{ ketal} = 1 \frac{\text{mole}}{\text{sec}}$

$1 \text{ ketal} = 6 \times 10^7 \text{ units}$

one mole of substrate transformed or product formed *per Second* under defined conditions

INHIBITION OF ENZYME ACTIVITY

$E_T = \downarrow$ $V_{max} = \downarrow$

Irreversible Inhibitor: Binds covalently (tightly) to enzyme
Enzyme activity is permanently lost.

1. Group-specific covalent modifying agents:

Serine:
- Diisopropylflurophosphate (DIFP)
- Phenylmethyl sulphonylfluoride (PMSF).

eg Trypsin
Protease cleaves after lysine or argine
Chymotrypsin

Cysteine:
Iodoacetate
Iodoacetamide
N-ethylmaleimide
Ag+, Hg2+, Pb2+

eg Caspase
Glyceraldehyde 3- Phosphate dehydrogenase

2. Affinity labels: binds to substrate binding site covalently

Tosyl Pheklylalanyl Chloromethyl Ketone (TPCK):

Binds covalently to histidine in substrate binding site of chymotrypsin. → protease cleaves after aromatic aa (Phe, Tyr, Trp)

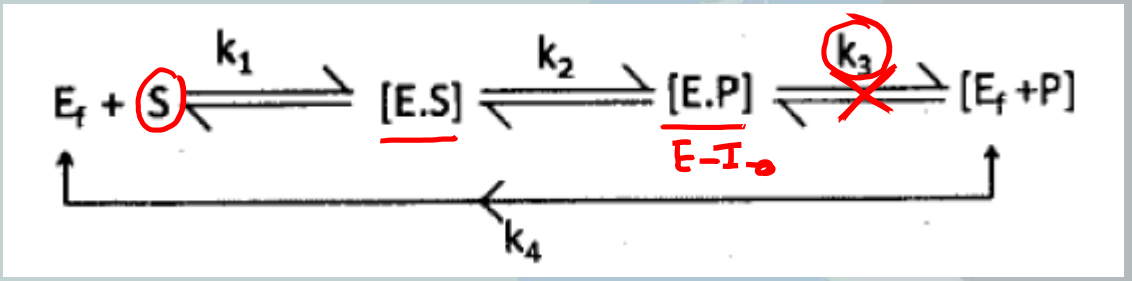
3. Transition state analogs: resembles transition state of substrate & binds covalently to substrate binding site

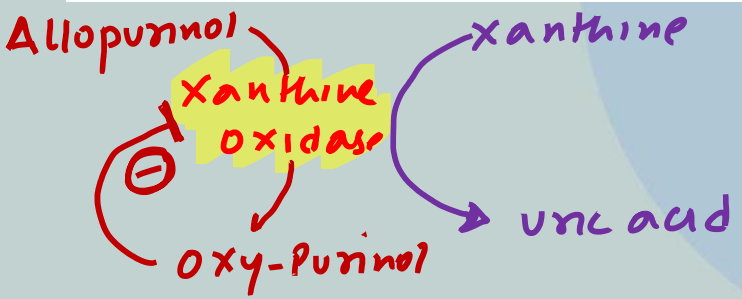
Pyrrole 2- carboxylate which resemble with transition state of proline and inhibits enzyme proline racemase.

4. Suicide inhibitors or *kcat* inhibitors

Eg. Allopurinol → Xanthine oxidase

Inhibitor directly donot bind to enzyme but If inhibitor are modified by enzyme than modified form binds covalently to enzyme.



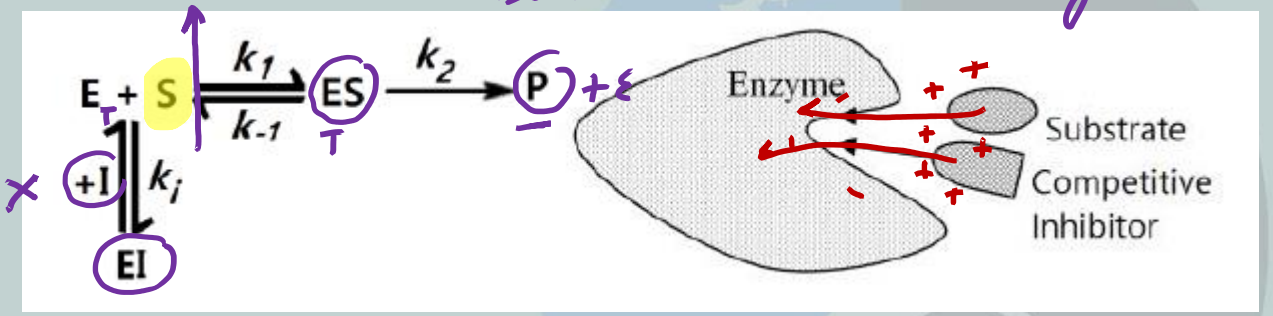


B. Reversible Inhibitors:

Binds non-covalently to enzyme

$E + I \rightleftharpoons EI$, reversed by decreasing conc^{r} of [I]

I. Competitive inhibition : Substrate & Inhibitor both compete for same substrate binding site.



ES or EI binary complex formation

- Inhibition can also be reversed by increasing substrate conc^{r}.

Effect on Vmax: unchanged

Effect on Km: Increase , Affinity decrease

Effect on MM and Lineweaver-Burke plot:

$\alpha = 1 + \frac{[I]}{K_i}$

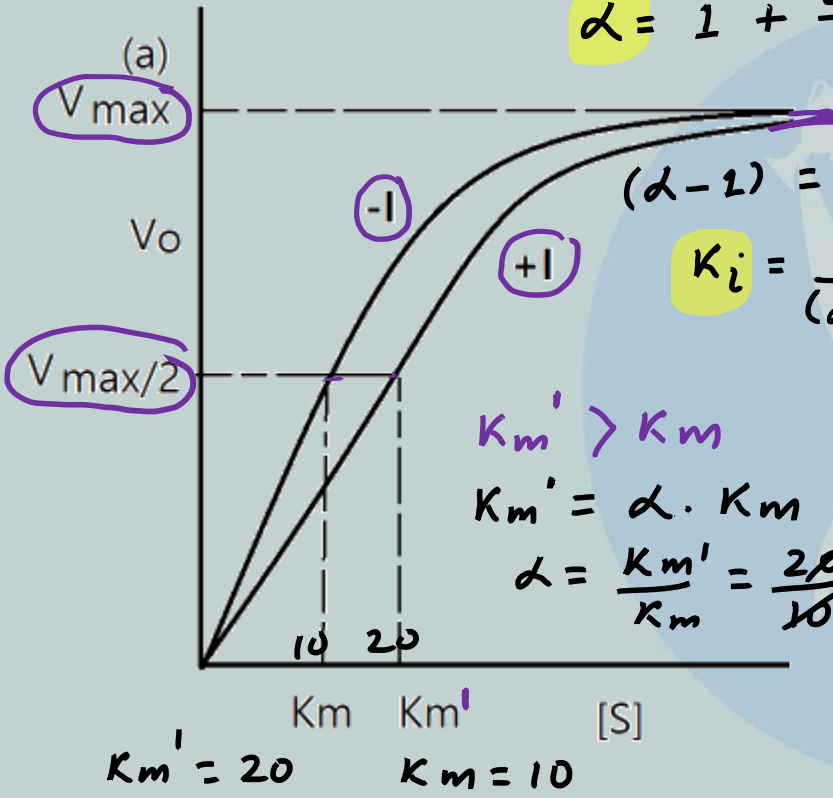
$(\alpha - 1) = \frac{I}{K_i}$

$K_i = \frac{I}{(\alpha - 1)}$

$K_m = \uparrow$

$V_{max} =$ No change

$\uparrow$ Slope $= \frac{K_m \uparrow}{V_{max}}$

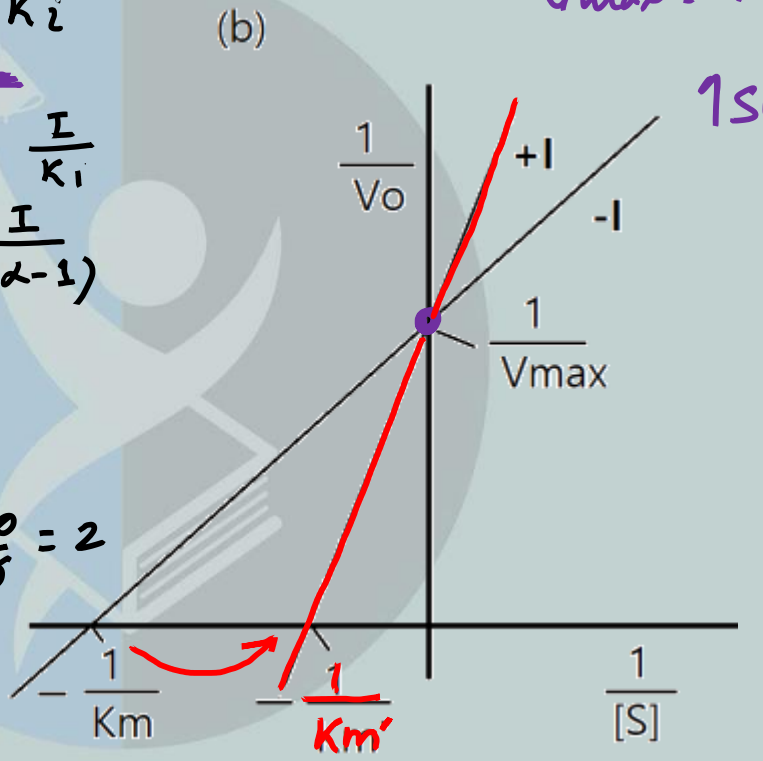


$K_m' > K_m$

$K_m' = \alpha \cdot K_m$

$\alpha = \frac{K_m'}{K_m} = \frac{20}{10} = 2$

$K_m' = 20$ $K_m = 10$



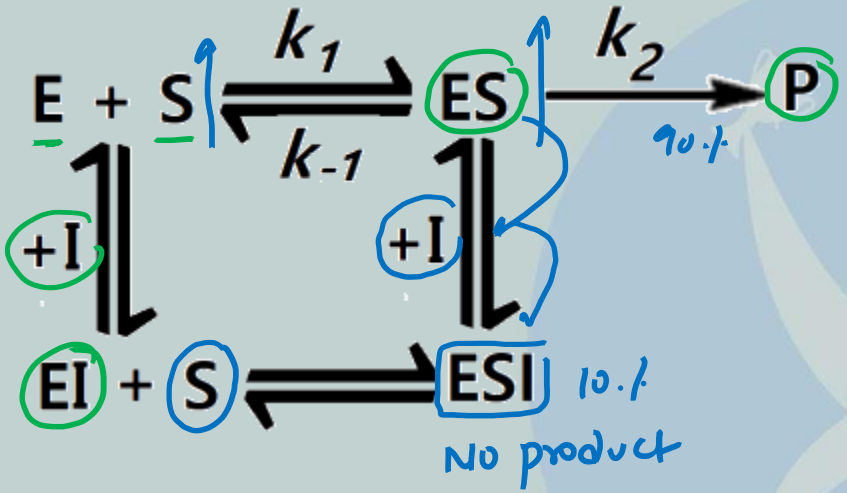
Examples of competitive inhibitors:

- Statin group drugs inhibits HMG CoA reductase (cholesterol biosynthesis)
- Malonic acid inhibits succinate dehydrogenase
- Sulpha drugs (sulfonamide) inhibits dihydropteroate synthase (DHPS) → folic acid biosynthesis
- Methotrexate and aminopterin inhibits dihyrdrofolate reductase
- ddI and AZT inhibit reverse transcriptase
- Ethanol inhibit alcohol dehydrogenase

H_2-folate → H_4-folate (THF)

↳ Treatment of methanol poisioning

2. Non-competitive inhibition



- Both EI & ESI complex are formed.

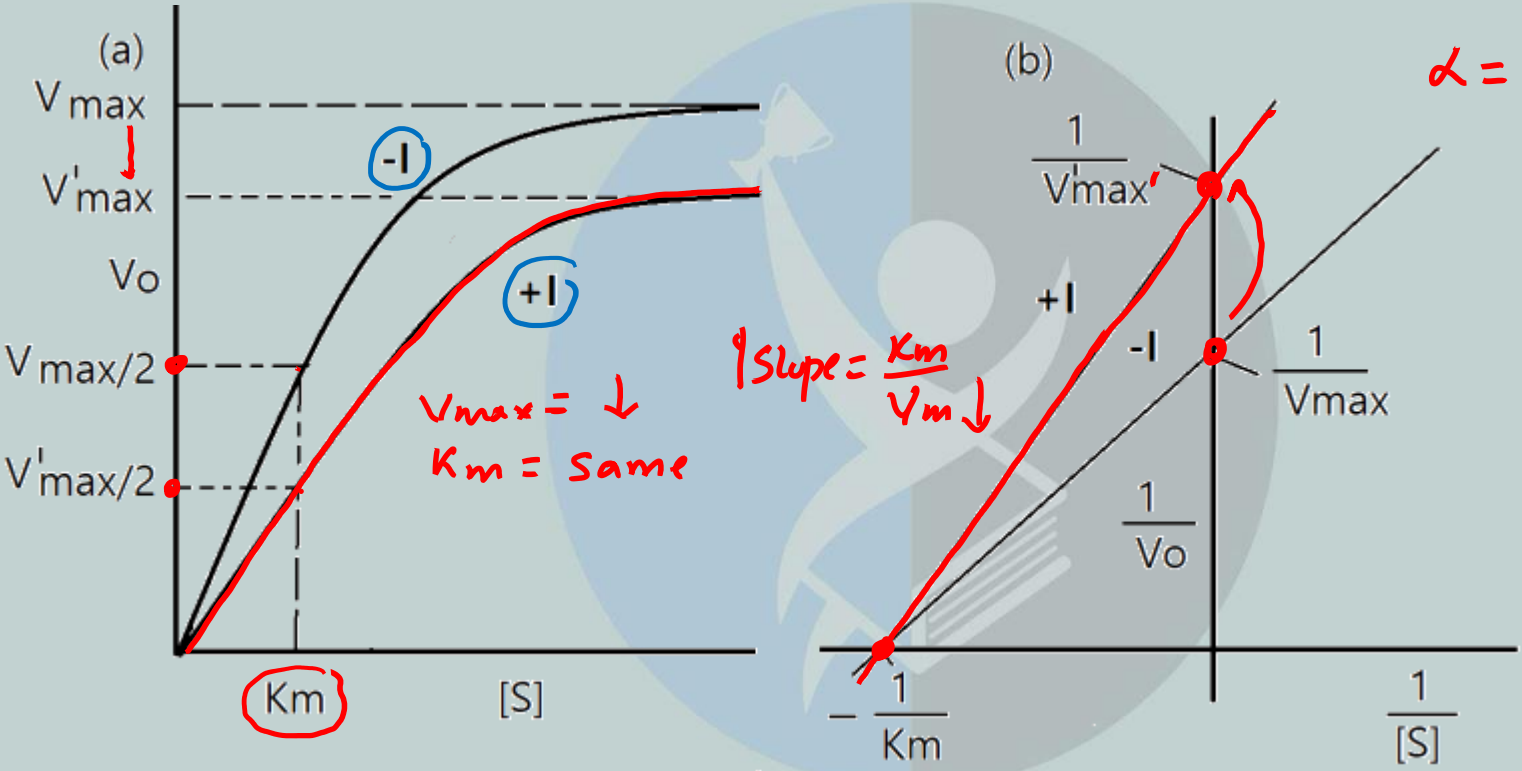
Effect on Vmax: Decrease

Effect on Km: No change, affinity of enzyme for substrate enzyme remain same

Effect on MM and Lineweaver-Burke plot:

$V_{max}' = \frac{V_{max}}{\alpha}$

$\alpha = 1 + \frac{[I]}{K_i}$



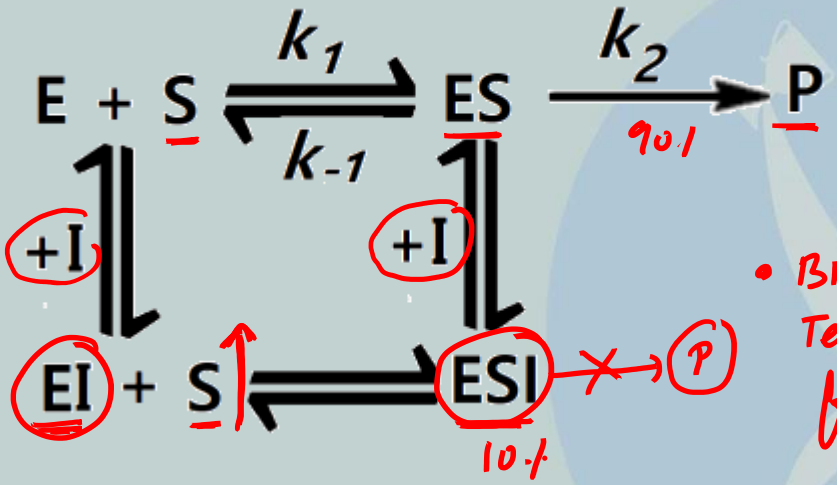
Examples of non-competitive inhibitors:

Cyanide inhibits *cytochrome oxidases*

complex IV of Electron transport chain

3. Mixed inhibition

Binding site of (S) & (I) are different.
Binding of (I) decrease affinity for (S) = Km ↑

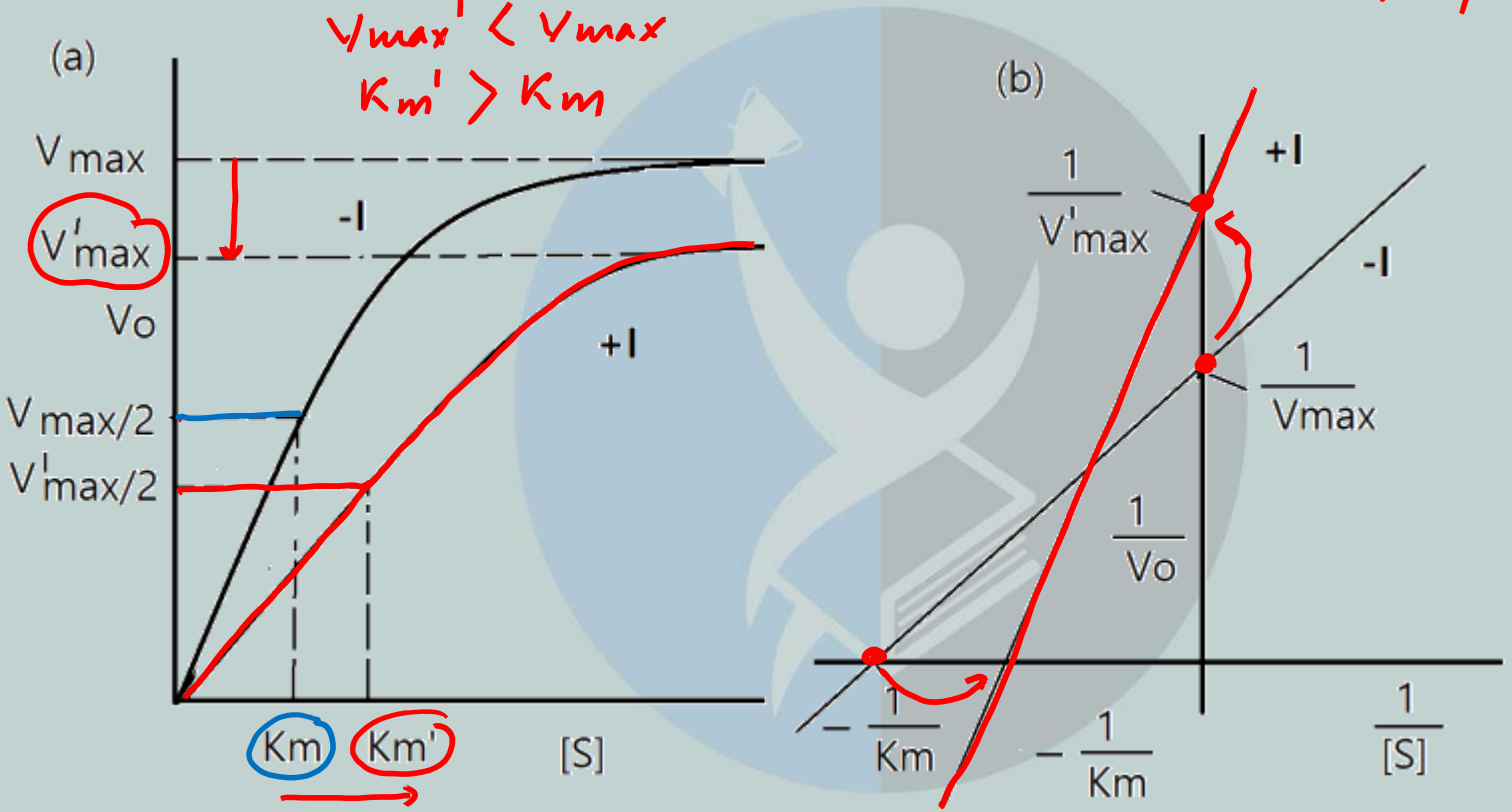


- Binary (EI) & Ternary (ESI) complex are formed

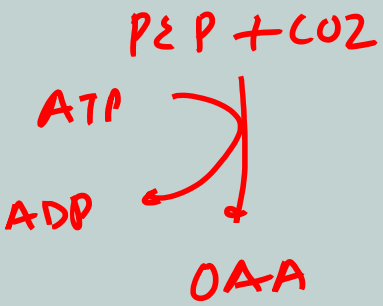
Effect on Vmax: Decrease
Effect on Km: Increase

Effect on MM and Lineweaver-Burke plot:

↑↑ slope = Km ↑ / Vmax ↓



Example: Phosphoenolpyruvate carboxykinase Inhibited by Genistein

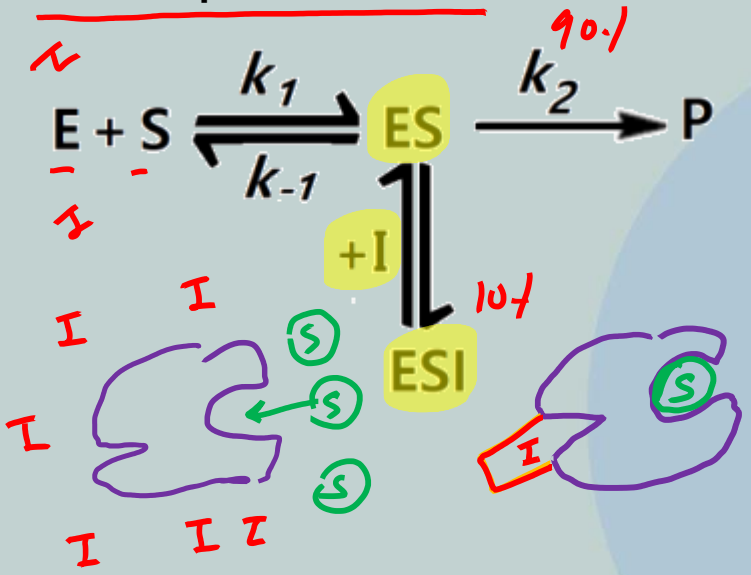


mixed inhibitor

Km = ↑

Vmax = ↓

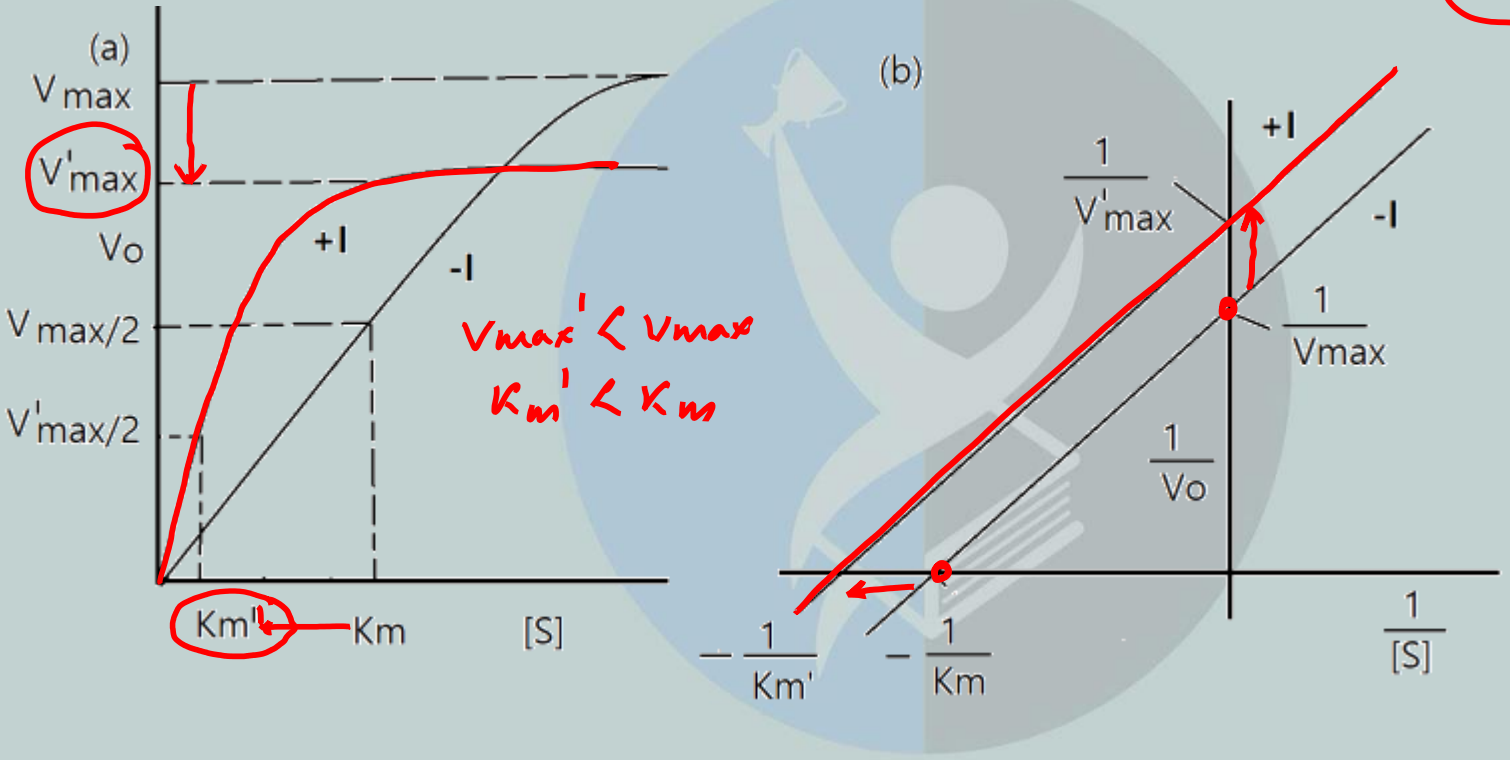
4. Uncompetitive inhibitors



- Binding site for substrate & inhibitor are different
- Inhibitor cannot bind to free enzyme
  No EI complex
- Presence of inhibitor favors binding of substrate (affinity = ↑)
  Km = Decrease
- Once ES complex forms, inhibitor binding site get exposed & inhibitor also binds to ES complex to form ESI ternary complex

Effect on Vmax: Decrease
Effect on Km: Decrease

Effect on MM and Lineweaver-Burke plot:



slope = Km/Vmax · 1/1

8/4 = 4/2

Examples of uncompetitive inhibitors:

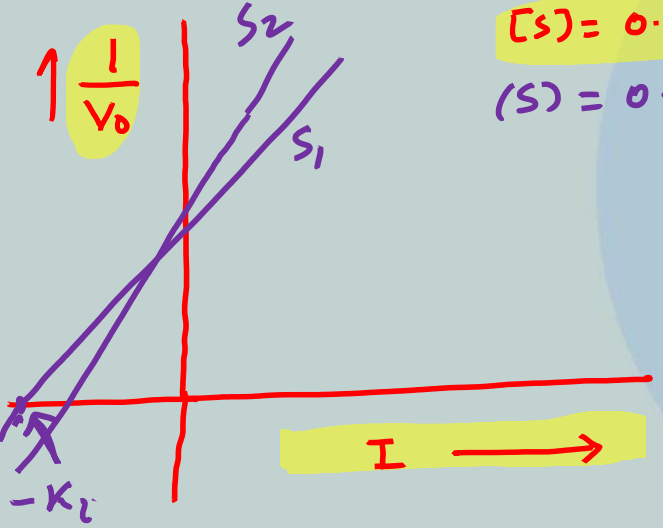
L- Phenylalanine inhibits ***Alkaline phosphatase***

Inhibition	Lineweaver Burk Equation
A. Competitive	1. $\frac{1}{v_o} = \left(\frac{\alpha K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$
B. Un-competitive	2. $\frac{1}{v_o} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$
C. Mixed	3. $\frac{1}{v_o} = \left(\frac{\alpha K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$

Dixon Plot

Important

Plotting between the reciprocal of the reaction velocity (1/v) against various concentrations of an inhibitor ([I]) at a fixed substrate (s) concentration.



[S] = 0.1 mM

(S) = 0.2 mM

x-axis = [I] ← Independent

y-axis = 1/V0 ← Dependent

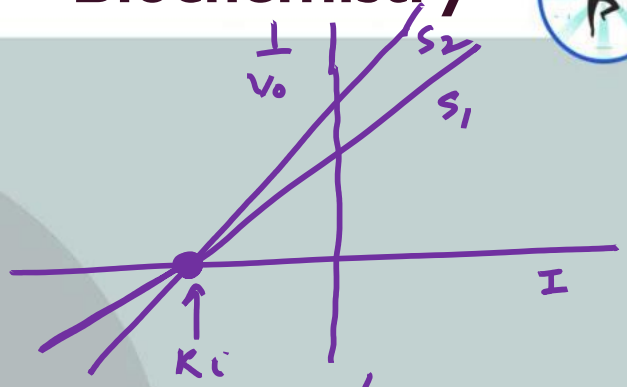
x-axis intercept

↓

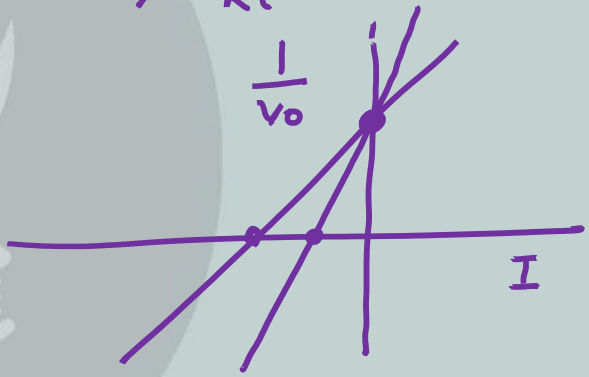
Ki

Not important

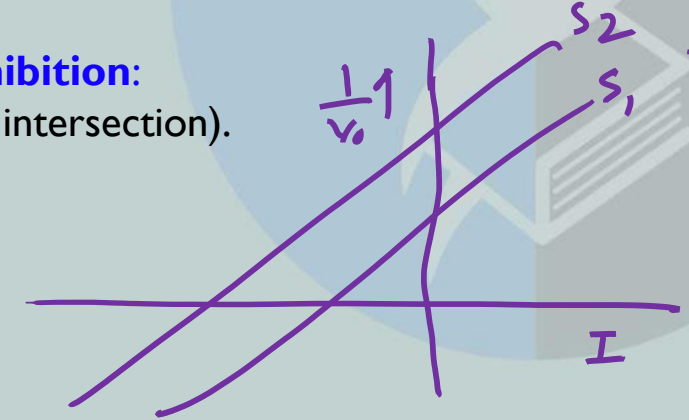
Competitive Inhibition:
Lines intersect on the x-axis (inhibitor concentration axis).



Non-competitive Inhibition:
Lines intersect on the y-axis (reciprocal velocity axis).



Uncompetitive Inhibition:
Lines are parallel (no intersection).



Apply Your Mind

Following statements are made about uncompetitive inhibition of an enzyme:

A. Inhibitor binds to free enzyme but not to enzyme- substrate complex.

B. Addition of uncompetitive inhibitor lowers the apparent Vmax and apparent Km of the reaction.

C. Apparent Km of the enzyme is increased but Vmax remains same

D. Inhibitor binds to free enzyme- substrate complex but not to free enzyme

Which one of the following option represents the correct combination of the statements ?

(1) B and C

(2) A and C

(3) B and D

(4) A and D

3

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